Induction of cell growth arrest and apoptotic cell death in human breast cancer MCF-7 cells by the COX-1 inhibitor FR122047

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Abstract. Non-steroidal anti-inflammatory drugs (NSAIDs), which inhibit the enzyme cyclooxygenase (COX), are known to have a potent anti-tumorigenic activity in various cancers. However, the responsible molecular mechanisms of COX inhibition in breast cancer cells remain to be completely elucidated. We examined the effect of the selective COX-1 inhibitor, FR122047 and the selective COX-2 inhibitor, SC791 on cell growth and apoptosis in human breast cancer MCF-7 cells which exhibited a high basal level of COX-1 expression. Compared to SC791, FR122047 treatment led to a distinct suppression of cell growth in MCF-7 cells. Upon FR122047 treatment, there were apparent increases in the ratio of Bax to Bcl-2, mitochondrial cytochrome c release, and apoptosis in MCF-7 cells. Our data showed that treatment of caspase-8 inhibitor could significantly suppress the cleavage of the effector caspase-7 and PARP in FR122047-treated MCF-7 cells which are caspase-3-deficient breast cancer cells, indicating that the induction of apoptosis by FR122047 is significantly dependent on caspase-8 activity in MCF-7 breast cancer cells. Our data suggest that the NSAID FR122047 may have an anti-cancer potential in breast cancer.

Introduction

Breast cancer is the most frequent malignancy in women globally and the second leading cause of death by cancer in females. Breast cancer cells develop through a multistep process such as hyperplasia, carcinoma *in situ*, invasive cancer and metastasis (1).

Cyclooxygenase (COX) is the principal enzyme in the synthesis of prostaglandins (PGs) from arachidonic acid and two isoforms of cyclooxygenase, COX-1 and COX-2, have been characterized. COX-1 is constitutively expressed in various tissues, whereas COX-2 is the inducible enzyme in response to certain stimuli including growth factors and pro-inflammatory cytokines (2,3). Several laboratories demon-strated that increased expression of COX-2 might be a contri-buting factor in breast cancer development (4-8). In addition to COX-2, COX-1 may also play a role in mammary carcino-genesis, and a report revealed that COX-1 selective inhibitor as well as COX-2 selective inhibitor led to inhibition of cell growth and metastatic capacity of mammary tumors (9). Actually, non-steroidal anti-inflammatory drugs (NSAIDs), which inhibit the enzyme cyclooxygenase (COX), have received attention because of their effects of prevention and treatment of tumor malignancy (3,8,10). Epidemiological studies have indicated that long-term use of NSAIDs is related to a reduced risk of breast cancer (11,12). In recent years, many reports have shown the anti-cancer activity of NSAIDs including cell cycle arrest, inhibition of cell proliferation, and induction of cell death (13,14). Because COX-2 is highly expressed in tumors and clearly enhances tumorigenesis, the focus has been on the use of COX-2 selective inhibitors in prevention of human cancer. However, much less attention has been paid to a possible role for the COX-1 inhibitors in breast cancer as compared to COX-2 inhibitor.

Apoptosis is the most characterized type of programmed cell death which is dependent on the caspase activity and is triggered by activation of both the mitochondrial (intrinsic) and death receptor (extrinsic) pathways, ultimately leading to cell death with membrane blebbing, cell shrinkage, and chromatin condensation (15,16).

Studies on the molecular mechanism of novel drug action are considerably essential for the development of chemo-therapy. FR122047, a selective COX-1 inhibitor was reported to have an analgesic effect in chemical nociceptive models and an anti-inflammatory effect in animal models of chronic inflammtion (17,18) but the anti-cancer activity of the chemical has not yet been identified. Here, we report that exposure of MCF-7 breast cancer cells to FR122047, a COX-1 inhibitor resulted in a dose-dependent cell growth arrest and apoptotic cell death. The compound inhibited the expression of the anti-apoptotic protein Bcl-2, resulting in an altered ratio of Bax to Bcl-2. Our data also indicate that FR122047 might activate major apoptosis signaling via caspase-8 activation in MCF-7 cells.

Materials and methods

Materials and antibodies. FR122047, a selective and potent inhibitor of COX-1 (17,18) and SC791, a selective COX-2 inhibitor (19,20) were purchased from Calbiochem (La Jolla,

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CA, USA). Stock solutions of FR122047 and SC791 were prepared in dimethyl sulfoxide (DMSO) and an equal volume of DMSO was added to the controls. 2',7'-Dichlorodihydro-fluorescein diacetate (DCFH-DA) and MitoTracker Red (mitochondrial dye) were from Molecular Probes (Eugene, OR, USA). Electrophoresis reagents and Bio-Rad protein assay kit were purchased from Bio-Rad. N-acetyl-L-cysteine (NAC) was purchased from Sigma (St. Louis, MO, USA). Pancaspase inhibitor (z-VAD-FMK), caspase-8 inhibitor (z-IETD-FMK), and caspase-9 inhibitor (z-LEHD-FMK) were from BD Biosciences (San Diego, CA, USA). Antibodies against Bcl-2, Bcl-xL, Bax, caspase-7, actin, cleaved poly (ADP-ribose) polymerase (PARP), and cytochrome c were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture. Human breast cancer MCF-7 cells (an estrogen receptor-positive cell line derived from an *in situ* carcinoma) were cultured in DMEM, supplemented with 10% heat-inactivated fetal bovine serum and 100 U/ml of penicillin/streptomycin (Life Technologies, Inc., Gaithersburg, MD, USA). MCF-10A cells (a non-tumorigenic epithelial cell line) were cultured in DMEM/F12, supplemented with 15 mM HEPES buffer, 5% horse serum, 10 μg/ml insulin, 20 ng/ml EGF, 100 ng/ml choleratoxin, 0.5 μg/ml hydrocortisone, and penicillin/streptomycin. Cells were maintained under a humidified atmosphere of 5% CO_2 at 37˚C.

RNA isolation and real-time RT-PCR. Total RNA was extracted from the cells using TRIzol reagent (Gibco-BRL, Gaithersburg, MD, USA) according to the manufacturer's protocol and the extracted RNA samples were subsequently treated with AMV Reverse transcriptase (Promega, Southampton, UK). Real-time PCRs were performed using specific primers for COX-1: forward, 5'-TAGAGATTGGG GCTCCCTTT-3' and reverse, 5'-AGGGACAGGTCTTGGT GTTG-3'; COX-2: forward, 5'-TGAGCATCTACGGTTTG CTG-3' and reverse, 5'-TGCTTGTCTGGAACAACTGC-3'; and GAPDH: forward, 5'-GGGAAGAGTCAACGGATTT GGTCGT-3' and reverse, 5'-GGGAATTGATTTTGGAGG GATCTCG-3'. Real-time PCRs were performed by a fluorescence detection method using the DNA Engine OPTICON® 2 system (MJ Research, Waltham, MA) and DyNAmo HS SYBR-Green qPCR kit (Finnzymes, Espoo, Finland).

Cell viability assay. Cultured cells were exposed to FR122047 and then their viability was analyzed by trypan blue exclusion assay. Briefly, an aliquot of the cell suspension was diluted 1:1 (v/v) with 0.4% trypan blue and the cells were counted with a hemocytometer. Results are expressed as the percentage of dead cells (ratio of stained cells vs. the control number of cells).

Flow cytometry analysis. Cells were detached from plates by the addition of 0.25% trypsin, washed in phosphate-buffered saline (PBS), fixed in 70% ethanol and stained with propidium iodide (PI) for 15 min at room temperature in the dark. Analysis of cells with sub-G0/G1 DNA content (subdiploid cells) was performed on 10,000 cells on a FACScan flow cytometer using the CellQuest analysis program (Becton-Dickinson), as described previously (21).
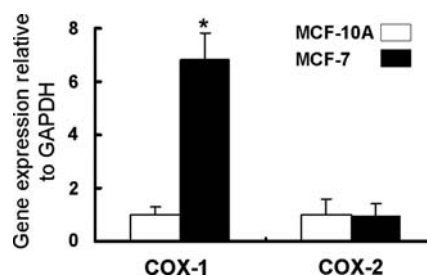

Figure 1. Expression of COX-1 and COX-2 mRNA in human breast normal and cancer cell lines. Total RNA was isolated and the mRNA level of COX-1 or COX-2 was measured by real-time RT-PCR. Expression levels relative to GAPDH are presented. The experiments were repeated independently at least three times. *P<0.05 vs. the control MCF-10A.
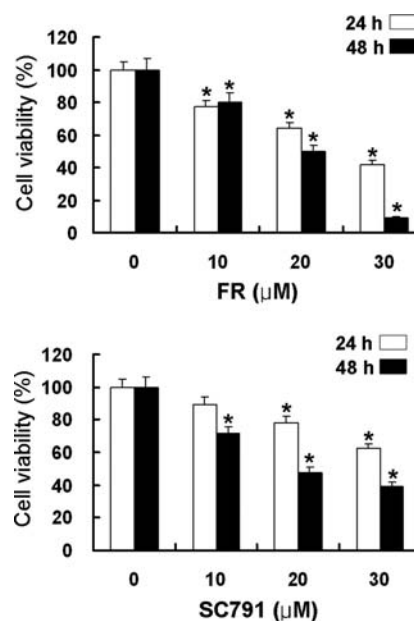

Figure 2. Growth inhibition after exposure to NSAIDs in MCF-7 human breast cancer cells. Cells were exposed to variable concentration of FR122047 (FR) or SC791 for 24 or 48 h. Cell viability of MCF-7 cells following drug treatment was measured by trypan blue exclusion assay. Values represent the mean ± SE from three independent experiments. *P<0.05 vs. the control.

Fluorescence microscopy. To investigate mitochondrial cytochrome c release, cells were seeded onto coverslips and allowed to attach overnight. Cells were exposed to 30 μM FR122047 for the desired time period at 37˚C, rinsed with PBS, and fixed with 3.7% paraformaldehyde in PBS for 20 min at room temperature. Cells were then permeabilized with 0.1% Triton X-100 for 15 min at room temperature, washed with PBS, and blocked in PBS containing 5% normal goat serum for 30 min at room temperature. Cells were treated with anti-cytochrome c antibody overnight at 4˚C, washed, and then incubated with FITC-conjugated goat anti-mouse secondary antibody for 1 h at room temperature. After washing with PBS, the cells were counterstained with MitoTracker Red (mitochondrial dye) to localize mitochondria and examined using a confocal microscope (Olympus, Japan).

Detection of intracellular ROS formation. For measurement of intracellular ROS levels, cells were incubated with 10 μM
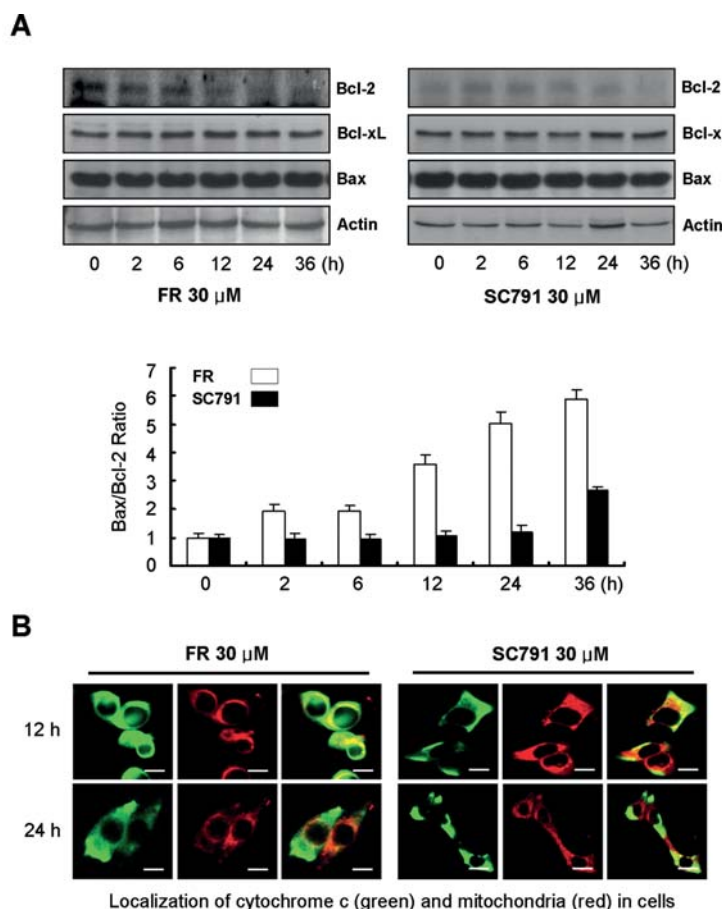
Figure 3. Effect of FR122047 and SC791 on expression of Bcl-2 family proteins and release of cytochrome c in MCF-7 cells. (A) Time-course expression of Bcl-2 family members after treatment of FR122047 or SC791. Cells were incubated with 30 μM FR122047 (FR) or 30 μM SC791 for the indicated time period and lysed. The expression of Bcl-2, Bcl-xL, or Bax was analyzed by Western blotting. Actin was used as loading control. Representative blots from three independent experiments are presented. The bottom panel displays the Bax/Bcl-2 ratio. (B) Immunocytochemical localization of cytochrome c and mitochondria in FR-treated MCF-7 cells. Cells were treated with 30 μM FR or 30 μM SC791 for 12 or 24 h, fixed, and stained with cytochrome c antibody (green, left panels) and MitoTracker Red (red, middle panels); overlay of staining patterns is shown in the right panels. Release of cytochrome c from the mitochondria into the cytosol was examined by confocal microscopy. Scale bar represents 10 μm. Representative images from three different experiments are presented.

DCFH-DA for 30 min at 37˚C in the dark, washed and suspended with PBS. Fluorescence-stained cells were analysed by fluorescence-activated cell sorting (FACS; Beckton-Dickinson FACScan) using CellQuest 3.2 software (Becton-Dickinson) for acquisition and analysis (22).

Western blot analysis. To prepare whole-cell extracts, cells were washed with ice-cold PBS and lysed in extraction buffer containing 1% Triton X-100, 100 mM Tris-HCl, pH 7.5, 10 mM NaCl, 10% glycerol, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 1 mM *p*-nitrophenyl phosphate, and 1 mM phenylmethylsulfonyl fluoride. After incubation on ice for 30 min, lysates were centrifuged and protein in the supernatants was quantified using the Bradford Protein Assay Reagent (Bio-Rad). An equal amount of protein was then separated on a 10-15% SDS-PAGE gel followed by electrophoretic transfer to nitrocellulose membranes and processed for Western blot analysis using appropriate antibody. Specific bands were detected by enhanced chemiluminescence system (Amersham Pharmacia Biotech, Piscataway, NJ, USA) as recommended by the manufacturer. The band density was quantified using NIH ImageJ software.

Statistical analysis. Data were expressed as means ± SE of three independent tests. The Student's t-test was conducted for testing statistical significance between groups and P<0.05 was considered significant.

Results

Expression of COX-1 and COX-2 in human breast cancer cells. The levels of COX-1 and COX-2 expression in MCF-10A cells (a non-tumorigenic epithelial cell line) and MCF-7 breast cancer cells were investigated by real-time RT-PCR. A very high basal level of COX-1 expression was observed in MCF-7 cells as compared to MCF-10A (Fig. 1), whereas no differences in the basal level of COX-2 expression were observed in MCF-10A and MCF-7 cells. These data indicate that a high basal level of COX-1 that is known to be constitutively expressed in almost all tissues may be associated with estrogen receptor-positive breast tumors.

Effect of COX-1 inhibitor and COX-2 inhibitor on growth of MCF-7 cells. The inhibitory effect of the COX-1 selective inhibitor FR122047 (FR) and COX-2 selective inhibitor
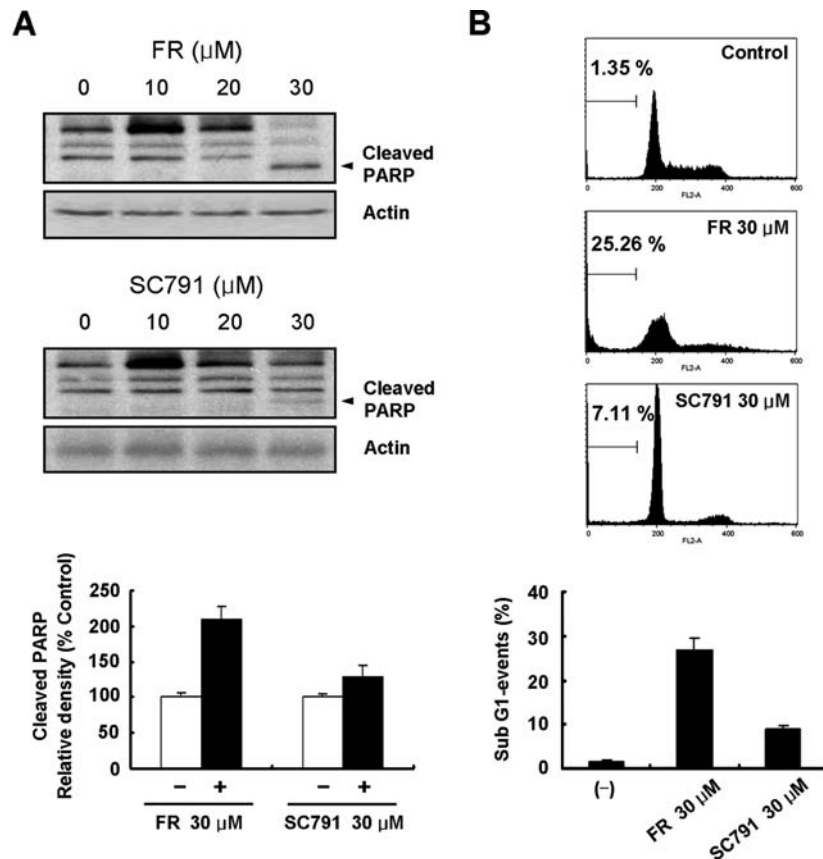
Figure 4. Induction of apoptosis by FR122047 treatment in MCF-7 cells. (A) Cells were treated with the indicated concentration of FR122047 (FR) or SC791 for 24 h, lysed, and proteins were separated by SDS-polyacrylamide gel electrophoresis. Western blot analysis was performed using antibody against cleaved PARP or actin (loading control). Representative data from three different experiments are presented. Bottom panel, results of densitometric analyses of the cleaved PARP levels. Values represent the mean ± SE from three independent experiments. (B) Percentage of subdiploid cells was determined by flow cytometry after treatment with 30 μM FR or 30 μM SC791 for 24 h. Representative results of three different experiments are presented.

SC791 on MCF-7 cell growth was first determined using the trypan blue exclusion assay. Both FR and SC791 had a significant dose- and time-dependent inhibitory effect on MCF-7 cell growth (Fig. 2). Treatment of FR exhibited a higher growth inhibitory effect than that of SC791.

Effect of FR122047 and SC791 on the levels of Bcl-2 family and mitochondrial cytochrome c release in MCF-7 cells. Changes in the ratio between pro-apoptotic and anti-apoptotic members of the Bcl-2 family, rather than the expression level of Bcl-2 family member alone, is crucial for drug-induced apoptosis (23,24). Therefore, we first analyzed the expression level of pro-apoptotic and anti-apoptotic members of the Bcl-2 family protein in COX-1 inhibitor FR122047 (FR)- or COX-2 inhibitor SC791-treated MCF-7 cells. Treatment of MCF-7 cells with FR122047 induced a rapid down-regulation of Bcl-2 with no change in Bcl-xL or Bax, resulting in an increase in the Bax/Bcl-2 ratio (Fig. 3A). Although MCF-7 cells exposed to SC791 also showed a slight decrease in Bcl-2 level, the change of the Bax/Bcl-2 ratio was just not significant compared to the case with FR122047 treatment (Fig. 3A). We next investigated the effect of FR122047 or SC791 on cytochrome c release by immuno-cytochemistry. The cytochrome c in MCF-7 cells treated with FR122047 for 12 h was mainly localized in mitochondria, as disclosed by a yellow staining due to the merging of

mitochondria-associated red fluorescence (MitoTracker Red) and green fluorescence derived from cytochrome c. Treatment of FR122047 for 24 h caused mitochondrial cytochrome c release to the cytosol in MCF-7 cells, which was evidenced by accumulation of cytochrome c-associated green fluorescence in the cytosol, whereas treatment of MCF-7 cells with SC791 did not trigger significant mitochondrial cytochrome c release.

Effect of FR122047 and SC791 on apoptosis in MCF-7 cells. Compared to SC791-treated cells, we revealed that PARP was more markedly cleaved in FR122047-treated MCF-7 cells (Fig. 4A). Treatment with FR122047 elicited a pronounced increase in the proportion of cells with sub-diploid DNA content but SC791-treated MCF-7 cells had a much lower proportion of cells with subdiploid DNA content (Fig. 4B).

FR122047 induces apoptosis in MCF-7 cells via caspase-8 activation independent of reactive oxygen species (ROS) production. In view of the known relationship between reactive oxygen species (ROS) generation and apoptosis, we examined whether ROS accumulation contributes to apoptosis in FR122047 (FR)-treated MCF-7 cells. MCF-7 cells treated with FR showed accumulation of intracellular ROS, which was abolished by addition of the anti-oxidant N-acetyl-cysteine (NAC) (Fig. 5A). However, treatment with
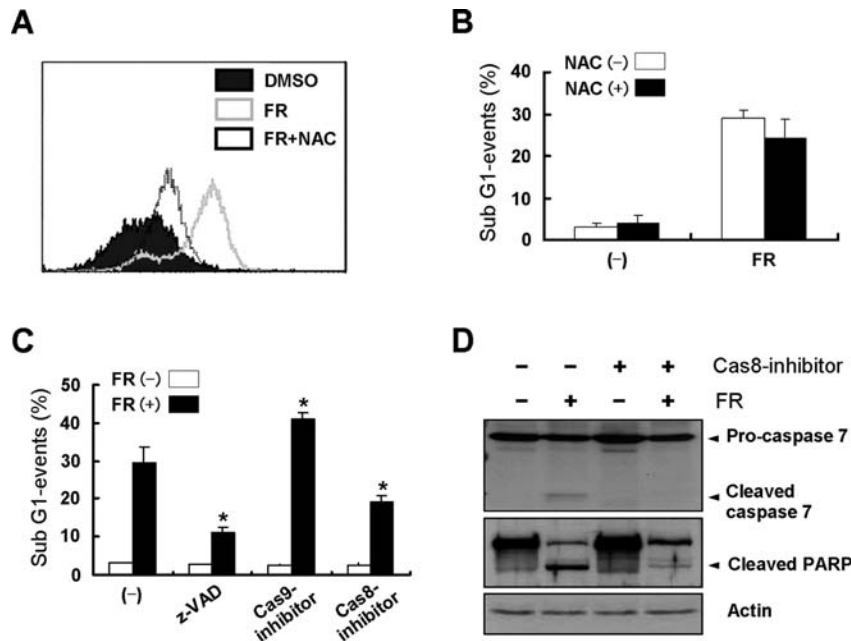
Figure 5. Induction of caspase-8-dependent apoptosis by FR122047 treatment in MCF-7 cells. (A) After treatment with 30 μM FR122047 (FR) or 2 mM NAC for 12 h, alone or in combination, cells were stained with the fluorescent probe DCFH-DA and intracellular ROS levels were measured by FACS analysis. Increased ROS level is indicated by an increase in fluorescence and shift of the curve to the right. Representative data from three different experiments are presented. (B) Cells were treated with 30 μM FR or 2 mM NAC for 24 h, alone or in combination, and analyzed by flow cytometry. Representative results of three different experiments are presented. (C) Cells were pretreated with 25 μM pan-caspase inhibitor z-VAD-FMK (z-VAD), 20 μM caspase-9 inhibitor z-LEHD-FMK (Cas9-inhibitor), or 50 μM caspase-8 inhibitor z-IETD-FMK (Cas8-inhibitor) for 1 h before the addition of 30 μM FR. Cells were incubated for another 24 h and the proportion of subdiploid cells was analyzed by FACScan. *P<0.05 vs. FR alone. (D) Cells were preincubated with 50 μM caspase-8 inhibitor z-IETD-FMK (Cas8-inhibitor) for 1 h prior to addition of 30 μM FR and incubation for an additional 24 h. Cell lysates were analyzed by Western blotting with antibodies against caspase-7, PARP, and actin (loading control). Images representative of the results of three different experiments are presented.

NAC did not significantly reverse FR-induced apoptosis in MCF-7 cells (Fig. 5B), indicating that ROS is not essential for FR-induced apoptosis. We next examined caspase activation during FR-induced cell death of MCF-7 cells. Addition of the pan-caspase inhibitor z-VAD-FMK or the caspase-8 inhibitor z-IETD-FMK significantly blocked the accumulation of a sub-diploid fraction in FR-treated cells but treatment with caspase-9 inhibitor z-LEHD-FMK did not (Fig. 5C). As expected, the caspase-8 inhibitor suppressed the cleavage of the effector caspase-7 and PARP in caspase 3-deficient MCF-7 cells (25), consistent with the caspase-8-dependence of apoptosis in FR-treated cells (Fig. 5D).

Discussion

Our present study showed that FR122047 (FR), a COX-1 inhibitor induced significant cell growth arrest and apoptosis in breast cancer MCF-7 cells. Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit the enzyme cyclooxygenase (COX) which convert arachidonic acid to prostaglandins (PGs). Since COXs are known to be highly expressed in tumors and to augment tumorigenesis, a number of studies have concentrated on investigating the possibility of using COX inhibitors for the prevention or treatment of cancer. Therefore, inhibition of PGs formation explain part of the anti-cancer effect of NSAIDs. Some studies have reported that inhibition of cell growth caused by treatment with a COX-2 inhibitor was reversed by addition of PGs (26,27). However, several lines of evidence suggest that NSAIDs could also regulate signal pathways through COX-independent mechanisms. For example, NSAIDs lessened cell survival in COX-deficient cell lines such as HCT-15 colon cancer cell line and C33A cervical cancer cell line (28,29) and have also been reported to induce anti-inflammatory effects and apoptosis through various signaling pathways independently of COXs inhibition (30-32). FR122047, a COX-1 inhibitor induced apoptosis in MCF-7 breast cancer cells by a mechanism independent of its ability to suppress PGs synthesis because exogenous PGE$_2$ had no effect on FR122047-induced apoptosis in MCF-7 cells (data not shown).

Celecoxib and other NSAIDs showed that drug-induced apoptosis could be caused through intrinsic pathway by cytochrome c release from mitochondria and activation of caspase-9 as well as extrinsic pathway by activation of caspase-8 (34-36). FR122047-treated MCF-7 cells exhibited a rapid down-regulation of Bcl-2, resulting in an increase in the Bax/Bcl-2 ratio. We also observed early apoptotic event, i.e., mitochondrial cytochrome c release, indicative of a well-established intrinsic apoptotic pathway in FR122047-treated MCF-7 cells (Fig. 3). Based on these observations, we proposed that caspase-9 could be involved in the mechanism of FR-induced cell death. Treatment of the caspase-9 inhibitor was unable to suppress, but rather enhanced FR-induced cell death, indicating that caspase-9 might be involved in another pathway specifically manipulating the typical apoptotic signaling pathway. However, additional experiments are required to analytically investigate this possibility and currently underway.

Activation of extracellular signal-regulated kinase 1/2 (ERK1/2) has been shown to incite apoptosis of HaCaT keratinocytes (33). Although FR122047 treatment induced activation of ERK1/2 in MCF-7 cells, an inhibitor of ERK1/2 activation failed to protect FR122047-induced cell death (data not shown), suggesting that the ERK1/2 may not be involved in FR122047-caused apoptosis in MCF-7 cells.

In summary, the present study showed that treatment of breast cancer MCF-7 cells with the NSAID FR122047 could lead to cell growth arrest and induction of caspase 8-mediated apoptosis independent of reactive oxygen species (ROS) production. Our data suggest that the NSAID FR122047 may have an anti-cancer potential in breast cancer.

Acknowledgements

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